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E. A. EDWARDS

M. W. RYTEL

R. L. HILDERBRAND

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NAVAL HEALTH RESEARCH CENTER

P. O. BOX 85122
SAN DIEGO, CALIFORNIA 92138

NAVAL MEDICAL RESEARCH AND DEVELOPMENT COMMAND

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IMMUNODIAGNOSTIC TECHNIQUES FOR BACTERIAL INFECTIONS*

Earl A. Edwards, M.S.[†]

Michael W. Rytel, M. D.^{††}

Richard L. Hilderbrand, Ph.D.[†]

Naval Health Research Center
P.O. Box 85122
San Diego, California 92138

and

The Medical College of Wisconsin
Milwaukee, Wisconsin 53226

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[†] Mr. Edwards is Head of the Biological Sciences Department, Naval Health Research Center, and Dr. Hilderbrand (LT MSC USN) was head of the Biochemistry Branch, Biological Sciences Department, Naval Health Research Center.

^{††} Dr. Rytel is Head, Section of Infectious Diseases, The Medical College of Wisconsin.

OVERVIEW

Immunodiagnostic techniques for detection of antigen or antibody immediately imply a variety of applications of immunological and immunochemical methods to the diagnosis of microbial diseases. Immunodiagnosis lends itself to the study of human diseases because of the sensitivity and specificity that specific immune sera (the immune response) provides. The early use of immunological diagnosis was to demonstrate circulating antibodies to disease producing microbial agents. This was done directly (Widal test) and by showing a rising titer between serum samples taken in the acute phase compared to those taken during convalescence. The data from such testing and their reliability in diagnosis of various microbial diseases has formed a solid cornerstone from which a much broader and more sophisticated technology has developed.

The initial application of precipitin reaction in the diagnosis of infectious diseases goes back to 1909 when Vincent and Bellot described detection of meningococcal antigen in cerebrospinal fluids of patients with meningitis.⁽¹⁾ Dochez and Avery in 1917 were the first to identify pneumococcal antigen in sera and urines of patients with lobar pneumonia.⁽²⁾

However, its been the data collected over the past 2 decades that has shown that specific antigen-antibody complexing can be a powerful tool in identifying either antigen or antibody. A thorough description of this reaction was made by Heidelberger and Kendall in the mid 1930's. Landsteiner and Pauling also played a major role in our understanding of the precipitin reaction in the late 30's and early 40's. That the precipitin reactions could occur and be visualized in gels was described by Ouchterlony and by Oudin in the late 40's.

The technique described by Ouchterlony has been widely used to identify multiple antigen and antibody systems. The method also allows one to show the immunological relationship between two antigens by the line confluence when two antigen preparations are used. The Ouchterlony technique has been extensively used to determine antigen or antibody purity, detect antigen in biological secretions and to quantitate either antigen or antibody when needed. Elik was one of the first to use the gel/precipitin reaction as a diagnostic tool to identify pathogenic strains of diphtheria.

This chapter will deal with several of the technologies that have been developed and have proven useful during the past decade in identifying either antigen or antibody. It will give more emphasis to identifying antigens because of the urgency of early identification of microbial agents for proper chemotherapy. In addition, the sensitivity of these techniques allows identification of picogram amounts of antigen, which are the levels that are frequently found in biological secretions during acute illnesses.

Techniques to be described will include the double diffusion test (Ouchterlony), counterimmunoelectrophoresis (CIE), rocket immunoelectrophoresis (Laurell), specifically sensitized Protein A-containing Staphylococcus aureus (Kronvall), radioimmunoassay (Catt), and enzyme-linked immunosorbent assay (Engvall).

DOUBLE DIFFUSION OR OUCHTERLONY TEST

The double diffusion test described by Ouchterlony makes use of the fact that soluble antigens and their antibodies will precipitate in a gel and the precipitate is permeable to all other antigens and antibodies that have no points of antigenic similarity with the precipitating pair. Thus, identical antigens or antibodies diffusing from two or more wells will form a precipitating band with an angle with one another that will fuse (lines of identity). By the same reasoning, antigens of different specificities will form an angle with one another that will cross (non-identity) or will partly cross and partly fuse (partial identity) (See Fig. 1 on pg 3).

The Ouchterlony test has been used to study microbial antigens and for the detection of antibodies. The test is relatively insensitive compared to other tests available and has not had broad clinical application but has proven very useful in research. It also suffers from the fact that it takes at least 24-28 hours for the results to become available. However, use has been made of the test for presumptive diagnosis and therefore a methodology for the macro double-diffusion test is described.

Materials:

1. Agarose
2. 0.15 M sodium chloride (saline)
3. Microscope slide (cleaned with Baboo and then with 95% ethanol, towel dry)
4. Well punch set (#51450 Gelman or individual well cutters 3.0 mm diameter)

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5. Pasteur pipettes or capillary tubes
6. Moist chamber

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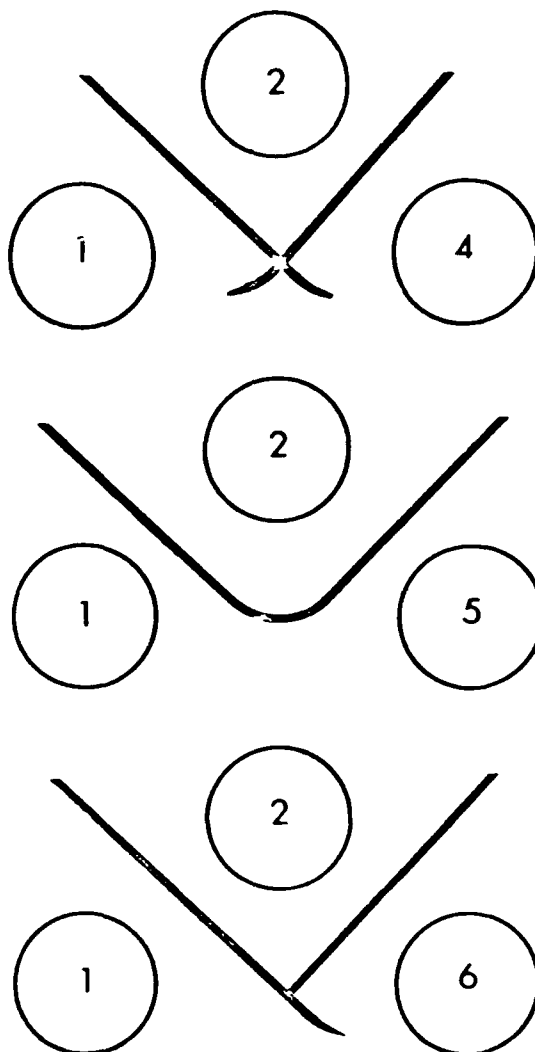


Fig. 1 A graphic preservation of patterns of precipitation that may be seen using the Ouchterlony technique to identify or compare antigens. Well #2 represents antiserum; wells 1, 4, 5, and 6 represent antigen preparations. Top: reaction of non-identity; middle: reaction of identity; bottom: reaction of partial identity.

Method:

1. Prepare 1% agarose in saline, bring to boil to dissolve agarose, overlay 2-2.5 ml onto a clean microscope slide.
2. Allow agarose to solidify (1 hr in moist chamber).
3. Punch desired number of wells in desired pattern. The distance between wells range from 205 mm. A circular pattern around a central well lends itself to many testing requirements.
4. Carefully remove punched plugs by suction.
5. Fill the central well with immune serum and the surrounding wells with test material (spinal fluid, serum, sputum, etc.).
6. Place in a moist chamber at room temperature for 24-72 hours.
7. Read the slides by use of oblique illumination. A hand lens 3-7X is highly recommended.
8. The precipitin bands may be easier to interpret after appropriate staining. Should this be necessary, the following steps are recommended.
 - a. Rinse or soak the slides (step 7 above) in a saline solution at room temperature for 2-3 days, making daily changes of the saline bath. This procedure removes the excess antigen and serum proteins.
 - b. Soak in distilled water for 1-2 hours.
 - c. Place the washed slides in either amido black or coomassie blud for 30 minutes.
 - d. Destain until precipitin bands are readily differentiated from the background.
 - e. Alternative to step c. Place slides at 37°C until completely dry. Proceed with step c.

Staining Solution:

Amido Black or Coomassie Brilliant Blue R-250	5.0 gram
Ethanol 95%	450.0 ml
Glacial Acetic Acid	100.0 ml
Distilled Water	450.0 ml

Destaining Solution:

Ethanol 95%	450.0 ml
Glacial Acetic Acid	100.0 ml
Distilled Water	450.0 ml

Caution: Unless the glass surface of the slides is carefully cleaned, the agarose may float off the slide during step 8.a. It is advisable to precoat the clean slide with a thin layer of a .2% agarose, allow to air dry and further super dry the slides for several days in a jar with CaCl_2 . This provides a bonding for the fresh agarose layer applied for double diffusion testing and reduces the chance of having the agarose float off the glass surface upon washing.

COUNTERIMMUNOELECTROPHORESIS (CIE)

Tiselius pioneered the separation of proteins by their moving boundaries which was carried out in a liquid phase system, however, his (Tiselius and Flodein) zone electrophoresis technology in the early 1950's set the stage for the immunoelectrophoresis techniques which were to follow in the late 50's and throughout the 60's. Immunoelectrophoresis was first described by Williams and Grabar in the mid 50's.⁽³⁾ Application of this technique allowed one to separate and identify mixtures of antigens and was especially used to study serum proteins and their mobilities in an electrical field. It became customary to carry out electrophoresis of serum proteins at pH 8.6 during this early stage of studying protein separation because most proteins are negatively charged at this pH and will migrate to the anode. The exception to this was the case of the three major classes of immunoglobulins. IgM and IgA remained either at the point of application or migrated toward the cathode. IgG, being made up of a heterogenous group of protein moieties, shows a broad range of movement, moving both anodal and cathodal. This (cathodal movement) was found to be due to the impurities in the gel (agaropectins) which have a strong negative charge. When in an electrical field, these "fixed" negative charges surround themselves with positive charges from the anode, thus moving the liquid buffer from the anode toward the cathode. This movement of buffer is called electroosmotic flow or endosmosis, and is effected by agar purity, concentration, thickness, buffer molarity and pH as well as by the voltage at which the electrophoresis is performed. These aspects will be addressed in greater detail below.

The endosmotic effect "carries" the relatively "neutral" charge of the immunoglobulins toward the cathode. This characteristic was used in developing the counterimmunoelectrophoresis (CIE) test as first described by Brussard in 1959.⁽⁴⁾ He called the test l'electrosynthese. The CIE test developed by Brussard has the advantage of using an electric current (and resulting endosmosis) as a migratory force, which increases the speed and sensitivity of the precipitin reaction. The technique was used sparingly until Gocke and Howe published their paper on its use to detect Australia antigen in 1970⁽⁵⁾ and a paper by Edwards in 1971⁽⁶⁾ to detect bacterial antigens. Since then, there has been widespread use of the technique to detect both bacterial, viral, fungal, protozoan, and various protein constituents associated with diseases.^(7,8,9,10,11,12) Current uses of CIE are summarized in Table 1.

Table 1. Current Uses of CIE in Infectious Diseases

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1. Detection of antigen in body fluids.
 2. Detection of antibodies.
 3. Prognostic usefulness, i.e., positive correlation between presence and amount of antigen and severity of illness and prognosis.
 4. Possible role of antigens in disease pathogenesis.
 5. Identification and typing of isolates in clinical microbiology laboratory.
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Although CIE is a variation of immunoelectrophoresis and agar gel diffusion, the various parameters involved seem much more critical. An illustration of a simple immunoelectrophoresis chamber is shown in Figure 2 (see page 7). Since the test has not been standardized with respect to variation in antigen composition, several parameters of the technique which influence sensitivity will be brought to the attention of the reader/user.

One of the critical points is to have the correct current/voltage so that the precipitin band is formed between the two wells (Figure 3, see page 7). A number of factors contribute to this success, i.e., well size, distance between wells, composition of agar, depth of agar, composition of buffer, concentration of antigen and antibody. These parameters will eventually be standardized as

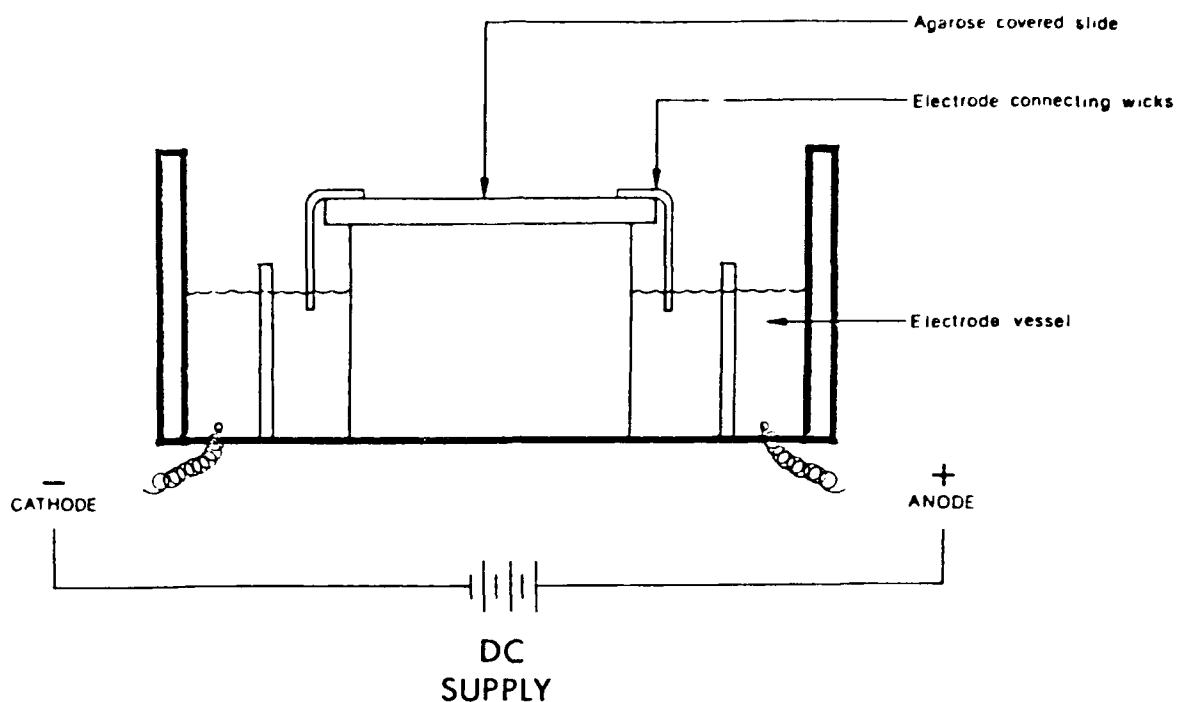


Figure 2. A simply designed electrophoresis chamber which is easily adapted to a single microscope slide, multiple slides, or Kodak glass plates.

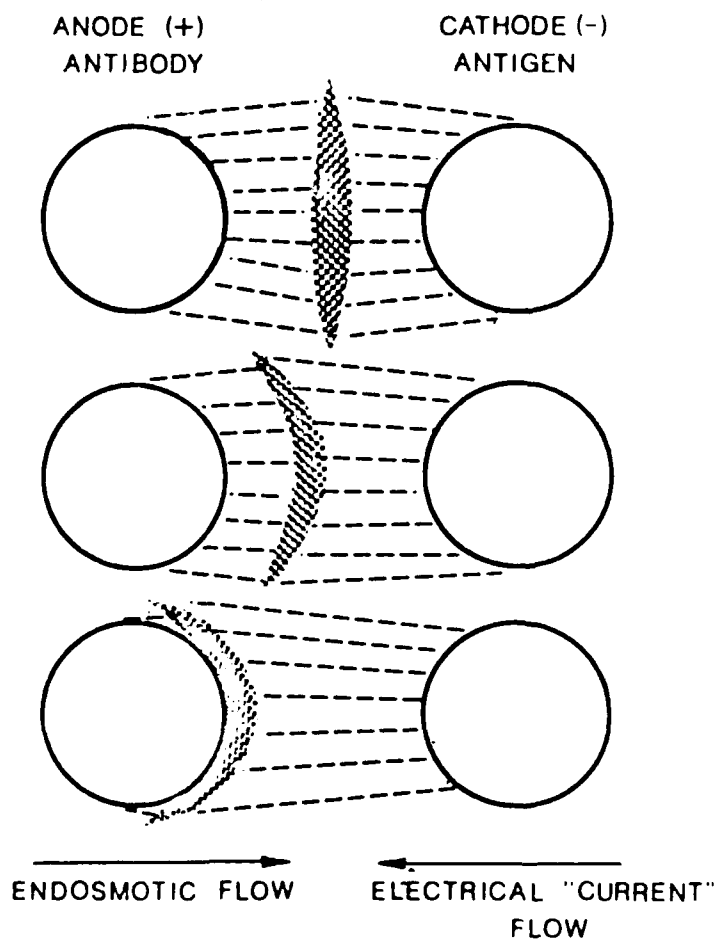


Figure 3. Antigen-antibody precipitin patterns observed in counterimmunoelectrophoresis. The upper pattern represents the optimal pattern, not often observed when testing clinical samples. The lower two patterns are frequently seen in clinical samples. The bottom pattern indicates antigen excess or a support medium with low endosmotic flow.

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commercially prepared reagents for CIE testing become available. However, until that time occurs, those points, which bear directly on the sensitivity of the test, will be discussed.

Well Size: For the antigens that have so far been successfully detected by CIE, a 3 mm diameter well separated by 2-4 mm (edge to edge) has proven satisfactory. This size well will hold from 5-7 μ l fluid. There are variations that can be substituted for this pattern. If the antibody used to detect antigen is relatively weak, the "antibody" well size can be increased to 5-8 mm in diameter, thus allowing a larger quantity of antibody to be used, at the same time, keeping the antigen well at 3 mm diameter and vice versa. Variations in well size patterns should be established with each lot of antisera to insure the sensitivity needed.

Distance Between Wells: It is recommended that when trying a "new" antigen-antibody system, a series of wells be spaced, starting at 2 mm (edge to edge) and increasing the distance between wells by 2 mm through 5 wells. Such a pattern will reveal an optimal range of distances which will give a precipitin band with the expected antigen concentration.

Composition and Depth of Agar: The less pure the agar, the greater the negative charge, and in turn, the greater the endosmotic flow. This can have catastrophic consequences on the CIE test. Agarose, although the quality varies from company to company and even from batch to batch, appears to give the most consistent results with the antigens so far detected. Each agarose batch should be checked for sensitivity. The depth of the agarose layer over the slide is another variable that has definite influence on the sensitivity of the test. Our experience indicates that agar thicknesses of 3 mm or greater are generally unsatisfactory. Optimal thickness ranges from 1-2 $\frac{1}{2}$ mm.

Buffer Composition: A wide variety of buffers have been used in CIE. However, barbital buffers seem to be the buffer of choice for most antigens. One exception is the use of a borate buffer when testing for pneumococci types 7 and 14.⁽¹³⁾ Barbital buffer (0.05-0.1 M) at pH 8.4-8.6, has been used successfully for most antigens since they are negatively charged at this pH. The concentration of the buffer in the agarose layer is of importance since the speed of migration of a given substance decreases as the ionic strength of the surrounding liquid increases. It should be pointed out that for buffers consisting of weak monobasic acids (such as barbital) the ionic strength (μ) is

equal to the concentration (M) of the salt. Some authors have used the μ and M interchangeably which has led to confusion. The buffer concentration has a direct influence on the endosmotic flow in relation to the impurities of the agar layer. It (endosmosis) is greater in less concentrated buffers and in buffers with a pH which favors the ionization of the COOH groups of the agarose, i.e., more alkaline buffers. It is greater with thick layers of agar than with thin layers of agar. These physiochemical variables are important considerations in selecting buffers and agar, both of which are critical to reproducibility and optimal immunological reactivity.

Concentration of antibody and antigen: The sensitivity of the CIE test, assuming all other parameters are optimal, depends upon the potency of the antisera. It has been the authors experience that this is the limiting factor in detecting small amounts of antigen. The test is also subject to prozone effects due to antigen excess. If a potent antiserum is used, prozones have not been observed in clinical material, even to the extent of 20 μ l/ml concentrations of meningococcal antigen in one spinal fluid examined. Our experience indicates that potent antisera is the critical ingredient. The authors have not observed prozones due to antibody excess even under experimental conditions. However, when an attempt is made to detect antibody in a clinical specimen, several antigen concentrations should be tried, as prozones with antigen excess have been seen.⁽¹³⁾

With potent antisera, pneumococcal and meningococcal antigen can be detected at from 1-2 μ g/ml concentration. Since only 5-7 μ l of body fluid (spinal fluid for example) is added to a well, this gives a sensitivity of 5-10 nanograms of antigen. This sensitivity has allowed several quantitative studies to be made in which the severity of illness or prognosis of the acute illness can be predicted. It has been found that the greater the antigen concentration, the more severe and prolonged is the course of recovery. Antigen quantitation in spinal fluids and blood sera has become an important adjunct to antigen detection due to the consistent empirical relationship between antigen level and prognosis. This aspect is discussed in greater detail below.

Table 2 (see pg 10) summarizes microbial antigens and antibodies which have been detected in body fluids or in vitro in the laboratories of the authors and other investigators. The three types of infections which have been studied the most extensively by CIE (besides hepatitis B), are those caused by meningococcus,

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Table 2. Detection of Microbial Antigens and Antibodies
by CIE in Various Body Fluids

Antigens (<u>in vivo</u>)	Detected in
Viral:	
HBsAg	Serum, tears, saliva, urine
Enteroviruses	Cerebrospinal fluid (CSF)
Bacterial:	
<u>Streptococcus pneumoniae</u>	Sputum, serum, urine, CSF, pleural, bullus, joint, peritoneal fluids
<u>Neisseria meningitidis</u>	
A, C, D, X, Y, Z	CSF, serum, joint fluid
<u>Haemophilus influenzae</u> type b	CSF, serum, subdural, joint fluid
<u>Pseudomonas aeruginosa</u>	Serum
<u>Klebsiella pneumoniae</u>	Serum, CSF, urine, pleural fluid
<u>E. coli</u> K1	CSF, serum
<u>Staphylococcus aureus</u> (teichoic acid)	CSF, pericardial fluid
<u>Streptococcus</u> group B	CSF
Antibodies	Detected in
Viral:	
Hepatitis B	Serum
Influenza A ₂	Serum
California encephalitis virus	Serum
Cytomegalovirus	Serum
Bacterial:	
<u>Serratia marcescens</u>	Serum
<u>Staphylococcus aureus</u>	Serum
<u>Mycoplasma pneumoniae</u>	Serum
Fungal:	
<u>Candida</u> sp.	Serum
<u>Coccidioides immitis</u>	Serum
<u>Histoplasma capsulatum</u>	Serum
<u>Actinomyces israeli</u>	Serum
<u>Aspergillus fumigatus</u>	Serum
Protozoan:	
<u>Trypanosoma cruzi</u>	Serum
<u>Entamoeba histolytica</u>	Serum
<u>Trichinella spiralis</u>	Serum
Antigens (<u>in vitro</u>)	Detected in
Viral:	
Plant viruses	Plant juice, tissue-culture fluid
Influenza A ₂	Tissue-culture, chick chorioallantoic fluid
Cytomegalovirus	Tissue culture fluid
Bacterial:	
<u>Streptococcus pneumoniae</u>	Broth culture extracts
<u>Streptococcus</u> groups A-F	Broth culture extracts
<u>Protopoccus magnus</u>	Broth culture extracts
<u>Klebsiella pneumoniae</u>	Broth culture extracts
<u>Bacteroides fragilis</u>	Broth culture extracts
Enterobacterial common antigen (<u>Escherichia coli</u> 014)	Broth culture extracts

pneumococcus, and hemophilus.^(6,10) Of the various clinical syndromes studied the highest diagnostic yield was obtained in meningitis caused by these three organisms (over 90% in most studies). The method has been found to be more specific and sensitive than the Gram stain, and more rapid than the culture of cerebral spinal fluid (CSF). It has an additional advantage over the latter in that it may remain positive in partially treated cases. In pneumococcal pneumonia, the results have been less satisfactory in that only approximately 50% of bacteremic cases have detectable antigen in their sera.⁽¹⁰⁾ This figure could be increased to 64% if both sera and urines (concentrated 20-fold by 95% ethanol precipitation at 5°C) were studied. Though CIE appears to be less sensitive⁽⁷⁾ than bacterial cultures in pneumococcal pneumonia, it continues to have the advantage of rapidity, and specificity in relation to sputum cultures. In this regard, current work on this disease focuses on detection by CIE of pneumococcal capsular antigens in sputa.⁽¹⁵⁾ The test was reported to be sensitive (100% [8 of 8] sputa from established cases of pneumococcal pneumonia were positive) and specific (saliva of 83 normal individuals was negative).

Two other areas of CIE application will be commended on briefly. One is the diagnostic application in antibody detection. The clinical situations where this has been found useful are summarized in Table 2. In general, presence of precipitin antibodies has been found to correlate with active infection.^(10,11,14) The specificity of the method could be increased by obtaining an antibody titer; a titer of $\geq 1:8$ has generally discriminated well between active infection and "serofast" status or presence of cross-reactive antibodies.⁽¹⁴⁾ An additional application of CIE has been in the identification and typing of microbial isolates obtained in vitro.^(9,16) In general, appropriate dilutions of sonicates of suspensions of bacterial cultures from plates or broth have been employed for study. This approach offers an additional diagnostic dimension in that it may still lead to a more rapid identification of pathogen, which was present in body fluids (blood, CSF, etc.) in insufficient quantity to yield enough antigen for direct detection of CIE.

Finally, one of the most intriguing "spin-offs" of the diagnostic application of CIE has been the demonstration of a direct relationship between the presence and the amount of antigen in body fluids and the disease morbidity and mortality. This relationship has been reported in a number of infectious diseases.^(2,17,22) Specifically, meningococcal disease patients with antigenemia had a higher

incidence of disseminated intravascular coagulation (DIC), shock and more prolonged duration of coma.⁽¹⁷⁾ In patients with pneumococcal infections, there was also a direct relationship between the quantity of circulating antigen and development of DIC, other complications, and mortality rates.^(18,19) In both infections, antigen levels correlated inversely with antibody response and levels of complement and its components.^(17,19) The interpretation of these findings is a matter of speculation, but they may signify formation of toxic immune complexes, or impairment of immunologic function which may have an adverse effect of resistance to the infection. Work is in progress in several laboratories to elucidate this problem.

Materials Needed

Apparatus required for CIE.

- 1) Variable DC power source with ampere and voltage meters.
- 2) Chamger with appropriately spaced electrode vessels.
- 3) Cleaned lantern or microscope slides. Wash with Baboo (or equal cleaner), towel dry, then wash in 95% ethanol, towel dry to remove all residue.
- 4) Filter paper for electrode wicks. (Eaton=Dykman #301 or Gelman SI290 were found to provide superior conductivity).
- 5) Approximately 3 mm diameter needles (Gelman well cutters, #51466).
- 6) Agarose (generally 1%) - there are several sources, they vary in quality from lot to lot. See references 23 & 24.
- 7) Buffer - a barbital buffer pH 8.6 has proven satisfactory for many antigen-antibody systems: However, the optimal buffer and pH should be established for each antigen to be identified.

The Test Procedure

The technique can briefly be described as follows:

A glass slide or lantern slide is covered with 1% agarose in buffer to a depth of 1-2½mm (2 ml per microscope slide/10-14 ml per lantern slide). After the agarose has solidified, two rows of wells are punched in the agarose, the rows being approximately 3 mm apart, edge to edge. The plugs are removed by suction, using a Pasteur pipet attached to a faucet aspirator. The prepared

plates are then placed in an electrophoresis apparatus for immediate use or can be stored in a moist chamber in the cold, for several (but not more than 4) days until needed. If the slides are not used the same day, a preservative (0.1% sodium azide final concentration) should be added to the agarose prior to layering over the glass slides to retard bacterial contamination. The slides are connected vessels by a paper wick. Pre-soak the filter paper to insure a satisfactory bridge with the agar layer. The sample to be tested (antigen) is placed in the wells on the cathode (-) side of the electrophoresis set-up and the antibody on the opposite wells (the anode side). The apparatus is connected to a power pack and run using from 3-6 mA per microscope slide or from 12-20 mA per lantern slide, as measured at the power source. For diagnostic detection of antibodies 25 mA has been employed.⁽¹⁴⁾ After one hour, turn off the power, remove the slides and observe for a precipitin band between the wells by using an oblique lighting effect, holding the slides against a dark background. A hand lens 3-7X is highly recommended. A simple and rapid method of intensifying the precipitin band is to soak the slide in 95% ethanol for 15-20 minutes or in physiologic saline for several hours. This provides greater contrast so weak reactions can be more readily observed, but does not alter the final results.

Quantitating antigen (previously identified): Two-fold dilutions of the specimen (1:1-1:128) are run against undiluted type-specific antiserum. Concurrently, for reference quantitation, purified type-specific antigen is also run; the following dilutions are employed: 25, 10, 5, 2.5, 1.0, 0.5, 0.1, 0.05 µg/ml.

Purified lyophilized antigen (for pneumococcus, see reference 25) should be reconstituted at concentrations no less than 5 mg/ml (stock) because of the possibility of polysaccharide absorbing onto glass or plastic at lower concentrations. The stock solution is stored at -20°C. Any left-over test dilutions below stock concentrations should be discarded.

Following is an example of quantitation of a pneumococcal (PNC) antigen:
Quantity of antigen (µg/ml) = specimen titer (dilution) x reference quantitation titer.

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Cathode (-)	Dilutions of Specimen	Anti- sera	Concentration of Purified PNC (µg/ml)	Anti- sera	Anode (+)
	1:1 0	0	25.0 0	0	
	1:2 0	0	10.0 0	0	
	1:4 0	0	5.0 0	0	
	1:8 0	0	2.5 0	0	
	1:16 0	0	1.0 0	0	
	1:32 0	0	0.5 0	0	
	1:64 0	0	0.1 0	0	
	1:128 0	0	0.05 0	0	
	1:256 0	0			

Results: µg antigen/ml serum = 16 x 0.5 µg/ml = 8.0 µg/ml.

Source of Reagents for Diagnostic CIE

Pneumococcal antiserum: omniserum, pooled antisera (groups A-I), specific types: Statens Seruminstitut, Amager Boulevard 80, DK 2300, Copenhagen S. Denmark (instruct them to mark customs slip as follows: "TSUS 437.76/Free Antitoxin"). Also available from Difco; however, Statens Seruminstitute reagents have been more reliable for the CIE test.

H. influenzae antiserum: Difco Laboratories, Box 1058A, Detroit, MI 48232; Hyland Labs, 330 Hyland Ave., PO Box 2214, Costa Mesa, CA 92626; and Burroughs-Wellcome. PO Box 1887, Greenville, NC 27834.

Meningococcal antiserum: Difco; and Burroughs-Wellcome.

Rocket Immunoelectrophoresis:

This test has not received wide clinical application. However, it seems to have the potential of being a sensitive method for antigen detection and quantitation. It has the added advantage of performing several tests on a single microscope or Kodak slide.

The test is basically a modification of the single-radial-diffusion method of Mancini but uses an electrical current to force diffusion of the antigen in a gel matrix containing antibody. The antigen reacts with antibody in the gel, giving ricket like zones of precipitation. Not only can antigen be detected but when known concentrations of antigen are added as controls, the rocket heights are proportional to the concentration of the antigen. Thus by plotting

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the height of the precipitation formed by the controls on arithmetic graph paper, the values for the unknown samples can be determined by simple extrapolation.

A limited number of different antibody impregnated agar plates are commercially available. These include IgG, IgA, IgM, C3; haptoglobin, albumin, alpha 1 antitrypsin and transferrin (ICL Scientific, 18249 Euclid St., Fountain Valley, CA 92708). Because of the technical difficulties in standardizing and preparing the plates with uniform agar layer thickness, it is recommended that no attempt be made (except under experimental conditions) to prepare the plates in-house for diagnostic use.

Specifically sensitized Protein A-containing *Staphylococcus aureus*.

The cell wall of most coagulase positive *Staphylococcus aureus* strains contain a protein, called protein A, which combines with the gamma globulin of most mammalian species. For many years it was thought that this was a classical antigen-antibody reaction which occurred because of "natural" antibodies due to frequent exposure to this common bacterium. However, Forsgren and Sjoquist found that the reaction was mediated by sites on the Fc part of immunoglobulin IgG and labelled the reaction a "pseudo-immune" reaction. The reaction between the staphylococcal protein A and the Fc part of IgG provides an example that a reaction can be due to IgG factors other than specific antigen combining sites. All known antibody combining sites are located on the Fab-fragment of the IgG molecule.

Inhibition experiments with isolated heavy and light chains of immunoglobulin IgG showed the protein A activity to be present only in the heavy chain preparations. When $F(ab')_2$, Fab, Fc and F'c fragments were studied, only the Fc fragment preparation gave inhibition of the precipitate formed between a myeloma globulin and protein A. These inhibition studies confirm the original studies of Forsgren and Sjöquist that the reactivity of normal human gamma globulin with staphylococcus protein A resides in the Fc part of the molecule and is analogous to investigations for rheumatoid factor which also reacts with the Fc fragment of the IgG molecule.

This spontaneous "uptake" of IgG by the coagulase positive *Staphylococcus aureus* by way of the Fc fraction, leaving the antigen binding Fab portion of the molecule free to combine with antigen has many practical applications. Kronvall showed that by sensitizing Protein A-containing *Staphylococcus aureus* with sub-

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agglutinating amounts of rabbit anti-pneumococcal typing serum, the staphylococcus would take on the specificity of the immune serum and be agglutinated only by the corresponding pneumococcal antigen. The antisera used must be specific as cross reactions occur as in any immunological method. The authors have found the sensitivity of the test to be greater than the capillary precipitation test for grouping streptococcus but not as sensitive as the CIE test. However, it is a rapid test in that a positive agglutination reaction occurs within 1-3 minutes.

The sensitized staph-reagent has been used to group/identify antigens from broth cultures and also to group/identify antigens directly upon the primary isolation plate, thus making it possible to give presumptive identification within hours after having received a test sample compared to 1-4 days by conventional methods. Until further data is gathered on the sensitivity and specificity of the test using antisera to a broad range of bacterial species, the use of the test is not to replace accepted standard procedures of microbial identification, but rather serve to augment these procedures and if/when so used, may provide early presumptive identification of an infectious agent.

MATERIALS AND METHODS

1. Staphylococcus aureus, Cowan I Strain, American Type Culture.
2. Trypticase Soy Broth (BBL) TSB
3. Clean 500 ml Erlenmyer flasks and 10x125 test tubes.
4. 250 ml polyethylene screw cap centrifuge tubes or if a 210 ml centrifuge head is not available, 50 ml polyethylene tubes can be used.
5. Centrifuge with 3-5000 rpm capabilities.
6. Phosphate Buffered Saline 0.03 M pH 7.3 (PBS).

PREPARATION OF STAPHYLOCOCCUS AUREUS

1. Check purity of stock culture by culting 18 hours, 37°C.
2. Pick a colony of pure Staphylococcus aureus and inoculate 2-5 ml tubes of TSB.
3. After 6-7 hours growth (log phase), gram stain for purity, if pure, pipette (aseptically) 1 ml into a 500 ml Erlenmyer flask containing

250 ml sterile TBS and inoculate a Blood Agar plate to recheck for purity.

4. Incubate flasks and plates at 37°C for 18-24 hours. (An incubator-shaker is preferred, however, static incubation can be used but the yield will be less than when using an incubator-shaker.)
5. As a safety precaution, inactivate the growth by either adding 0.5 ml betapropiolactone, mix and allow to stand at room temperature for 2 hours or by heating at 56°C for 2 hours.
6. Centrifuge to pellet the bacterial cells and wash 3 times with PBS.
7. Make a 10% suspension of the sediment in 0.5% formaldehyde in PBS. Allow to stand at room temperature for 3 hours with occasional shaking.
8. Heat suspension at 80°C in a hot water bath for 1 hour.
9. Wash with PBS three times and store at a 10% suspension in PBS containing 0.1% sodium azide.

SENSITIZING STAPHYLOCOCCUS AUREUS

1. Remove stock 10% suspension S. aureus from refrigerator. Mix thoroughly to insure complete suspension and to break up small clumps that may have developed during storage.
2. Transfer 1 ml (fractions or multiples thereof) of the 10% stock suspension to a clean test tube.
3. Add 0.1 ml of immune serum, mix. (Ratio of immune serum to stock cells should remain at 0.1 to 1.0 ml respectively.)
4. Allow to "sensitize" for 1-2 hours at room temperature. Shake periodically (every 15 minutes) to promote optimal sensitization.
5. Pellet staph, wash pellet at least once with PBS and resuspend to 10 ml with PBS.

TEST PROCEDURE TO IDENTIFY COLONY^{*} OF ORGANISMS FROM PRIMARY ISOLATE

1. Make at least 2 parafin rings around suspected colonies. A wire

^{*}Group A,B,C,D streptococci; meningococci group A, C,Y; Salmonella A,B,C,C1, D, and Shigella sonnei and flexnerii, pneumococcus types 3,4,6,7,8,9,11,12,13,14, 18,19, and 23 have been shown to give a positive test with polyvalent (omniserum) sensitized staph.

- ring 12 mm in diameter has been suitable for this purpose. The parafin has to be hot so a good seal is made with the agar surface.
2. Add a drop of sensitized staph reagent to one ring around a suspected colony(s) and a drop of unsensitized staph reagent around a similar suspected colony.
 3. Rotate the petri dish in a to and fro pattern for at least 1 minute. Preliminary observations can be made using a dissecting scope. Continue the rotations for 3 minutes before final reading. Positive aggregates usually form by 1-2 minutes. The negative (unsensitized staph) should remain negative. (Continue mixing of the staph reagent over the colony is very important for development of visible aggregates.)
 4. Observe for large aggregates using the dissecting microscope. Other methods of observing aggregation of staph reagent have not proven satisfactory.
 5. Record results.

PROCEDURE FOR IDENTIFYING ORGANISMS FROM A BROTH CULTURE

1. Transfer suspected organisms to 1-5 ml TSB, or in the case of Salmonella, inoculate Dulcitol-Selenite enrichment broth, incubate 12-18 hours or until growth is evident.
2. Transfer 1 drop of the growth onto a marked area of a microscope slide and a drop of uninoculated growth medium to another area of the slide, add 1 drop of specific sensitized staph reagent to both the test and control test area.
3. Mix thoroughly with an applicator stick. Rotate slide for 1-2 minutes.
4. Observe for agglutination. The control should remain negative throughout the observation period.

ENZYME-LINKED IMMUNOSORBENT ASSAY

Introduction

In 1971, Engvall and Perlmann⁽²⁶⁾ and van Weeman and Schuurs⁽²⁷⁾ independently reported the use of enzymes conjugated to antibodies for the detection and assay of biological molecules. This technique was the outgrowth of the use of enzyme labeled antibodies to detect and localize cellular antigen in light- and electron-microscopy studies^(28,29) and is an alternative to the radioimmunoassay (RIA) method. Enzyme-linked immunosorbent assay (ELISA) has been designated variously as enzyme- or enzyme-immunoassay (EIA) and immuno-enzymatic assay. Enzyme-linked immunospecific assay (also ELISA) has been proposed by Engvall as a title with a broader range of application.

The procedures used in enzyme immunoassays are directly analogous to the techniques developed for RIA and are of the same range of sensitivity due to the amplification provided by the enzyme. In comparison with radioimmunoassay, ELISA uses only spectrophotometric or visual detection rather than the expensive instrumentation necessary for quantitation of radioactivity. In addition, with ELISA the need for special training and procedures for handling and disposal of reagents is obviated. RIA and ELISA have been used in similar manners for serodiagnosis (e.g. 30). However, RIA has not been widely applied in diagnostic microbiology although direct and indirect methods for bacterial identification have been developed.⁽³¹⁾ RIA has also been used for identification of staphylococcal enterotoxins A, B, and C, and for clostridial toxin.^(32,33) The principle use of ELISA has been in serology by the use of conjugates directed against a particular antibody.

Several different ELISA procedures have been used to measure antigen and/or antibody. In the "competitive" assay for antigen, labeled antigen and unlabeled antigen compete for a limited quantity of antibody bound to a solid support. The excess antigen is eluted and either the free or bound enzyme is quantitated and related to standard concentrations of antigen.

In the "homogenous" assay an enzyme bound to a hapten is inactivated when an antibody specific for that hapten is present. The addition of free unlabeled hapten, as either a standard or test, will compete for the antibody binding

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site freeing the hapten and thus the enzyme in proportional quantities. The enzyme can then be quantitated, producing an assay which requires no separation step. This procedure works only for haptens.

For "immunoenzymometric" assay antigen is reacted with excess labeled antibody. Excess solid phase antigen is then added to remove any free labeled antibody and separated. The labeled antibody-antigen complex remaining in solution is then quantitated.

Another method used for quantitating antibody is performed by attaching antigen to a solid support and adding the antibody to be measured. Following incubation an excess of enzyme labeled antibody with specificity for the first antibody is added followed by incubation and quantitation of the bound enzyme.

The method described in this chapter is an example of a "sandwich" assay (see Figure 4, pg 21). The method requires that the antigen (material to be detected) have at least two antibody binding sites.

Summary of Methodology for "Sandwich Assay"

1. Specific antibody is adsorbed to a solid support (polystyrene or polypropylene) and the support washed.
2. Appropriate antigen in standards or tests is complexed to the specific antibody adsorbed on the solid support and non-bound material removed by washing.
3. The same specific antibody as in 1. which has been chemically conjugated to an enzyme is incubated with the antigen. Non-bound material is again removed by washing.
4. Substrate is added to allow colorimetric quantitation of the enzyme label bound to antigen. This value is proportional to the quantity of antigen present in standards allowing one to quantitate the color produced over the range of standards and to relate this to the concentration of antigen in test materials.

If the second antibody is added unlabeled and a third incubation with excess labeled antibody with specificity for the second antibody rather than for the antigen is added, the assay is termed a "double sandwich". This last technique has the advantage that one labeled antibody can be used to quantitate a number of specific antibodies from a single species.

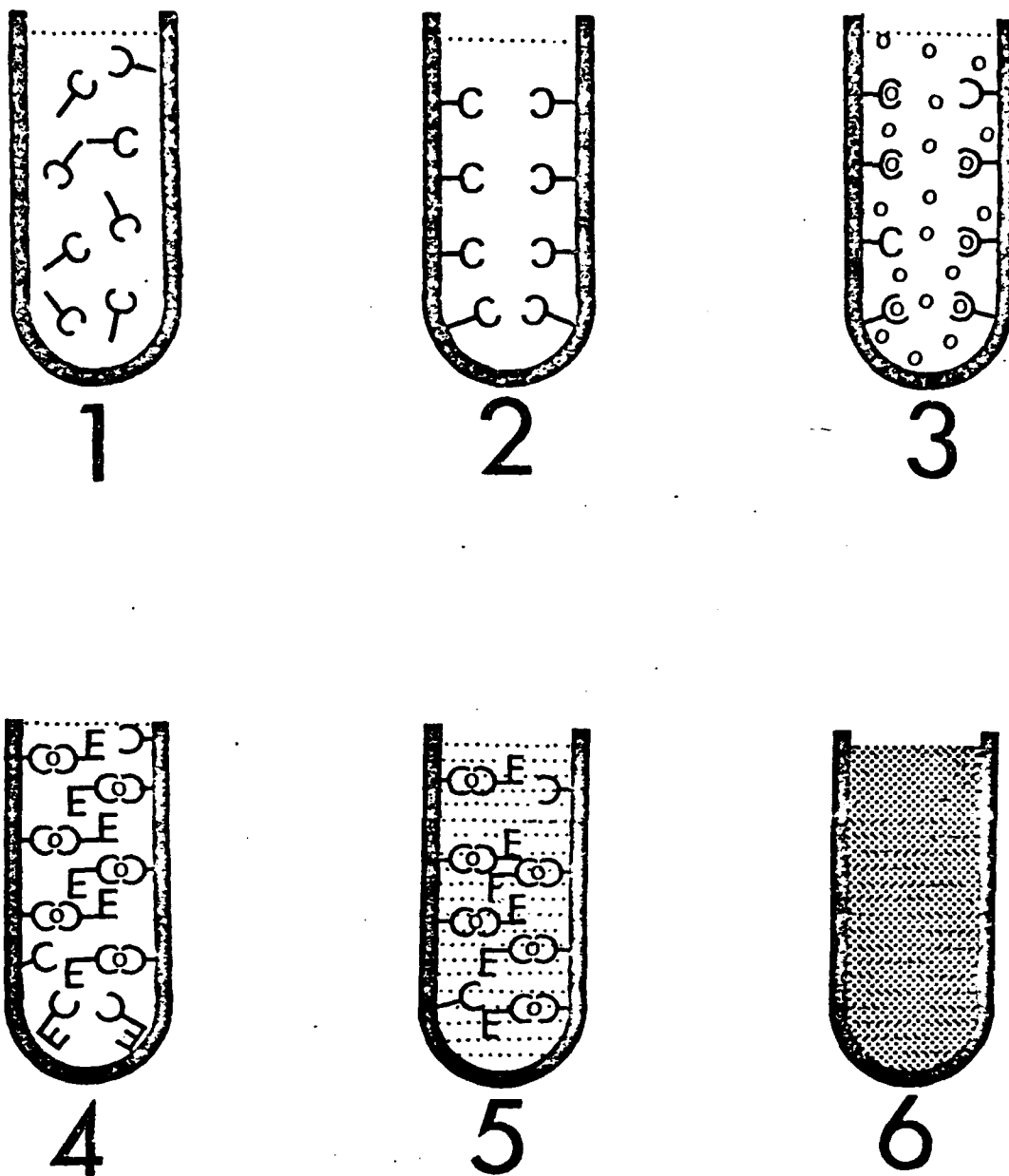


Fig. 4 Schematic of "sandwich" ELISA. 1) Specific antibody is added and 2) absorbed to solid support. 3) Antigen to be measured is added, bound by the specific antibody, and the unbound antigen washed out. 4) Specific antibody conjugated to an enzyme is added and bound to the antigen remaining on solid support antibody. 5) Enzyme substrate is added and 6) the optical density of the product assayed visually or spectrophotometrically.

Below is a procedural outline for commonly used methods of adsorption, conjugation, and immunoassay. Alternative procedures are included, however, a master list of required materials has not been presented. Materials needed will include polystyrene or polypropylene tubes (12x75 mm, Sarstedt), Tween 20 (J. T. Baker), Glutaraldehyde (Sigma), and either Alkaline Phosphatase (Sigma) and p-Nitrophenyl phosphate (Sigma) or Horseradish peroxidase (Sigma) and 2,2'-Azino-di[3-ethyl-benzthiazoline sulfonate (6)] (ABTS, Boehringer-Mannheim).

PROCEDURE

I. Adsorption of Specific Antibody to Solid Support

(Select one of the two methods).

- A. The most frequently used solid support for serodiagnostic methods has been polystyrene⁽³⁴⁾ in the form of either tubes or plates. The procedure for the adsorption of protein to polystyrene tubes is as follows:

Dilute specific antibody material to be adsorbed just prior to use with 0.1 M Na_2CO_3 (pH 9.8). For dilution see Section on titration of solid phase antibody. Add 1.0 ml of this solution to a polystyrene tube (12x75 mm) and allow it to set at 37°C for three hours. An additional period (overnight) at 4°C may also be used, but adsorption seems essentially complete within 3 hours. Wash the tubes three times with gentle swirling with 2 ml 0.9% NaCl containing 0.05% Tween 20 (Saline-Tween). The Saline-Tween wash as used throughout this discussion is intended to reduce non-specific adsorption of antigen or antibody in subsequent steps without removing the solid phase antibody. If nonspecific adsorption is still a problem the wash procedure may incorporate 10% aged human serum or 4% BSA or 0.5 M NaCl with the 0.05% Tween 20. The tubes prepared with adsorbed antibody can be stored at 4°C for several weeks.

- B. The following method using polypropylene tubes has been reported as superior to the polystyrene method because glutaraldehyde is used to link the protein to the solid support. There is apparently little or no "leakage" of protein during wash or assay procedures.

Incubate polypropylene tubes (12x75 mm, Sarstedt) with 1.0 ml freshly prepared 0.1% glutaraldehyde in 0.1 M carbonate buffer (pH 9.0) for 3 hours at 56°C and wash thoroughly with deionized water. Incubate tubes for 20 hours at 4°C using 1.0 ml of an appropriate dilution of antiserum in 0.02 M phosphate 0.9% NaCl (pH 7.2) and 0.02% NaN_3 as preservative. The diluted antiserum can be left in the tubes at 4°C and the tubes used for up to eight weeks. ⁽³⁵⁾

II. Conjugation of Enzyme-label to antibody.

A. Alkaline Phosphatase (AP). ⁽²⁶⁾

Combine 1.0 mg of the specific antibody to be conjugated and 3.0 mg of the AP (Sigma, Type VII) in 0.2 ml total volume of 0.1 M phosphate buffer (pH 6.8). If either material contains $(\text{NH}_4)_2\text{SO}_4$ dialyze prior to conjugation procedure. Add 8.0 μl of 25% aqueous glutaraldehyde which has been diluted with phosphate buffer (1:4, v:v) for a final glutaraldehyde concentration of approximately 0.2%. Allow to set for 2 hours at room temperature, dialyze exhaustively at 4°C against 0.05 M tris(hydroxymethyl) aminomethane-hydrochloride (Tris-HCl), pH 8.0, and chromatograph on Sephadex G-200 (1.5 x 90 cm) in the same buffer. The conjugate will elute in the void volume and unconjugated material will be retained. The void volume fraction of AP conjugates can then be stabilized by addition of 4% human serum albumin and 0.02% NaN_3 stored at 4°C and used for immunoassay. If necessary, a centrifugation at 10,000 xg for several minutes may be used to remove aggregates. Another procedure for higher specific activity AP is also published. ⁽³⁶⁾

B. Horseradish peroxidase (HRP).

The procedure of Nakane ⁽³⁷⁾ allows greater efficiency of coupling of HRP to either IgG or to F(ab')_2 fragments than either a one or two step glutaraldehyde procedure. The procedure is not as simple as the glutaraldehyde but has provided better results in our work.

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Add 0.1 of 1% 1-fluoro-2,4-dinitrobenzene in absolute ethanol to 5 mg of HRP (Sigma, Type VI) dissolved in 1.0 ml of freshly made 0.3 M sodium bicarbonate (pH 8.1). Mix gently for 1 hour (room temperature) and add 1.0 ml of 0.06 M NaIO_4 in water. Mix gently for 30 minutes at room temperature. Add 1.0 ml of 0.16 M ethylene glycol in water and mix gently for 1 hour at room temperature. Dialyze this solution against 3 x 1 liter changes of 0.01 M sodium carbonate (pH 9.15) at 4°C. Add 5 mg IgG in 1 ml of carbonate buffer to the dialysate and mix gently for 2-3 hours at room temperature. To the IgG-HRP conjugate add 5 mg NaBH_4 and allow to stand at 4°C overnight. Dialyze this at 4°C against 0.02 M phosphate 0.9% NaCl (pH 7.2) (PBS) and remove any precipitate by centrifugation. Purify the conjugate by Sephadex G-200 column (1.5 x 90 cm) chromatography in PBS and take the void volume fraction. HRP conjugate can be stabilized with 1% BSA and stored in aliquots at 020°C without azide.

For procedure A. or B. a purified antibody fraction (such as 45% $(\text{NH}_4)_2\text{SO}_4$ fraction, an IgG fragment F(ab')_2 or an antibody purified by affinity chromatography) can be used for conjugation to improve sensitivity or specificity.

III. Immunoassay

A. Alkaline Phosphatase.

1. Wash the antibody coated tubes 3 times with 2 ml Saline-Tween.
2. Dilute standards and serum to be tested with 0.02 M phosphate, 0.9% NaCl, 0.05% Tween 20 and 0.02% NaN_3 , pH 7.2 (PBS-Tween). Add 1 ml of diluted standard or serum to each tube and incubate at about 30°C with mixing for 3-6 hours. Wash 3 times with Saline-Tween.
3. Dilute enzyme-antibody conjugate to optimal concentration with 0.05 Tris-HCl pH 8.0 and add 1 ml of conjugate to each tube. Allow to incubate with gentle mixing at about 30°C 3-16 hours. Wash 3 times with Saline-Tween.
4. Add 1 ml of 0.05 M sodium carbonate 1 mM MgCl_2 buffer (pH 9.8) containing 1 mg/ml p-Nitrophenyl phosphate.(36)
5. Incubate for a suitable time at room temperature (e.g. 30 minutes). Add 0.1 ml 1N NaOH to Stop the reaction and read at 400 nm. Plot increase in absorbance units vs. standards.

B. Horseradish Peroxidase (HRP)

1. Wash the antibody coated tubes 3 times with 2 ml Saline-Tween.
2. Dilute standards and serum to be tested with 0.02 M phosphate, 0.9% NaCl, 0.05% Tween 20, pH 7.2 (PBS-Tween). Add 1 ml of diluted standard or serum to each tube and incubate at about 30°C with mixing for 306 hours. Wash 3 times with Saline-Tween.
3. Dilute enzyme-antibody conjugate to optimal concentration with PBS-Tween and add 1 ml of conjugate to each tube. Allow to incubate with gentle mixing at about 30°C 3-16 hours. Wash 3 times with Saline-Tween.
4. Add 1 ml of ABTS reagent. Make this by adding 2 mg ABTS per 50 ml of 0.03 M citric acid, 0.04 M phosphate, pH 4.0. Immediately before use add 25 μ l of 30% H_2O_2 per 50 ml of prepared ABTS solution.⁽³⁸⁾
5. Incubate at 25°C for 10 minutes. Stop the reaction by addition of 0.05 ml 0.3% NaN_3 or by pouring off solid phase. Read optical density at 415 nm.

or alternatively for HRP

4. add 1 ml of substrate for peroxidase. This is prepared by adding 80 mg of 5-aminosalicylic acid to 100 ml deionized water at 70°C and cooling to room temperature. Directly prior to use adjust the pH to 6.0 with 1 N NaOH. To 9 ml 5-aminosalicylic acid add 1 ml of 0.05% Hydrogen peroxide.⁽³⁹⁾
5. Incubate at room temperature for exactly 60 minutes stopped by addition of 0.1 ml 1 N NaOH and read at 449 nm. Plot increase in absorbance units vs. standards.

IV. Dilution of Reagents

A. Titration to solid phase Antibody

To determine the dilution of antiserum to adsorb to the solid support to give maximal uptake of antigen, take serial 10 fold dilutions of antiserum up to 10,000 fold. Adsorb each dilution to the solid support as described. Then add the highest concentration of antigen which you expect to use in your assay to each dilution. Complete the assay as described above and select the solid phase antibody dilution which gives the greatest enzymatic activity.

B. Titration of Conjugated Antibody

To a set of tubes coated with the optimal concentration of specific

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antibody add the highest concentration of antigen which you expect to use. Incubate, wash, and complete the assay using dilutions (e.g., 1:40, 1:80, 1:160, 1:32, 1:640) of conjugated antiserum. Select the optimal dilution.

DISCUSSION

Developmental work increasing the sensitivity and improving separation methods of ELISA is moving rapidly ahead. In addition, improvements are being made in methods for determination of the end point of the assay and in automation. The application of enzyme kinetic analysis will aid in both automation and quantitation. ELISA faces the limitations which are common to any immunoassay in that the characteristics of the antibody determine sensitivity and specificity of the assay. Also, for initial assays, the components of the assay must be prepared and titrated to maximize the assay. Some technical problems are that the titrations of antibody and conjugated antibody must be completed for each new lot of tubes that is used, each new lot of antiserum and each new conjugate preparation. Antibody specificity and accuracy in measurement are of utmost importance in quantitation, due to the amplification provided by the enzyme.

In the selection of an enzyme, there are several factors which are of prime consideration.

1. The end product of the enzymatic reaction should be visible to the naked eye if ELISA is to be used for screening purposes, absorb in either visible or ultraviolet regions for spectrophotometric quantitation, or be fluorescent for fluorimetric determination.
2. The end product should have a high molar extinction coefficient.
3. The enzyme should have high specific activity.
4. The enzyme should be stable, easily obtainable, and not inhibited by constituents of the material to be tested.

Alkaline Phosphatase (AP) meets the the enzyme requirements for labeling and has been used frequently. AP is preferable to peroxidase for quantitative ELISA and can be stored at 4°C with 0.02% NaN_3 as preservative for several months. The substrate of choice for AP is p-Nitrophenyl phosphate. Since inorganic phosphate is an end product of the enzymatic reaction, one must avoid the use of phosphate buffers in the enzyme substrate solution which could alter the rate of hydrolysis of the substrate.

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Another frequently used enzyme with the same range of sensitivity as AP is horseradish peroxidase (HRP). HRP is a smaller molecule than AP and is thus useful in histochemistry because diffusion into tissue is enhanced. For glutaraldehyde conjugation with HRP, one may use a one step (as described here for conjugation of AP) or two step procedure.⁽⁴⁰⁾ HRP has the unusual characteristic of reacting with only one glutaraldehyde molecule which limits self coupling of HRP and allows excess glutaraldehyde to be dialyzed out. The "activated" HRP can be added directly to the IgG which increases coupling efficiency and decreases self coupling of the second protein. Due to simplicity and time, the one step procedure has been used most frequently. The HRP conjugates can be stored sterilized by filtration and frozen in small aliquots.

The substrate for HRP is H_2O_2 , however, there are a number of chromagens for HRP of which one can be selected for use with the substrate. Diaminobenzidine (DAB) is available as a chromagen but is more useful for histochemistry than for ELISA because the end product forms a polymeric compound which is insoluble in aqueous solution. 5-Aminosalicylic acid (5-AS) and O-Phenylenediamine (OPD) are good substrates but OPD is difficult to handle because it is somewhat sensitive to light. The chromagen of choice for greatest sensitivity is ABTS.

Beta-Galactosidase is a much larger enzyme that is also being used. This enzyme can be used with 4-methylumbelliferone to produce a fluorescent product which has proven to be the most sensitive ELISA assay to date.⁽⁴¹⁾

APPLICATION

ELISA has been used to detect antibodies to viral, bacterial, and parasitic antigens and bacterial antigens themselves. The simplicity and speed of the technique has led to an application in veterinary medicine for detecting pathogenic organisms in meat on line at slaughterhouses. Saunders et al. have developed capability for multiple screening of serum antibodies to bacterial pathogens. Initial reports identify Trichinella spiralis, Brucella abortus⁽⁴²⁾ and hog cholera⁽⁴³⁾ and show good correlation with previous methods and in most cases improved sensitivity. In addition, Saunders has reported the detection of Staphylococcal Enterotoxin A to a level of 3.2 ng toxin/ml of prepared food product in 1-3 hours test time.⁽⁴⁴⁾ Ruitenbergh has used ELISA for the identification of T. spiralis infection in pigs at the slaughterhouse.⁽⁴⁵⁾

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A second major application has been in disease screening for infectious disease in humans in field studies in remote areas. The characteristic of the assay that gives a yes or no answer by visual or crude colorimetric methods makes this application possible. Voller et al⁽⁴⁶⁾ have demonstrated the potential of the test in a seroepidemiological study in an area of Columbia where malaris is endemic. Using microtiter trays and using visual and spectrophotometric methods they demonstrated the serological differences in the population of endemic area and area where antimalarial measures have been taken. Voller et al.⁽⁴⁷⁾ and Ruitenberg and Buys⁽⁴⁸⁾ have applied ELISA micro-techniques to the seroepidemiological studies in African trypanosomiasis.

Other applications have included serodiagnosis of syphilis⁽⁴⁹⁾ and schistosomiasis,⁽⁵⁰⁾ the detection of streptococcal M protein antibodies⁽⁵¹⁾ and detection of hepatitis B surface antigen.⁽⁵²⁾

SUMMARY

Enzyme-linked immunosorbent assay (ELISA) is a recently developed technology that has sensitivity and accuracy in the range of radioimmunoassay (RIA) technology and has the same specificity. The enzyme label can be stable for months and the enzymatic activity can be quantitated spectrophotometrically. The general features for radio- and enzyme-immunoassay are the same. However, in ELISA, separation is usually provided by the use of a polystyrene or polypropylene tube or microtiter trays as a solid support. Alkaline phosphatase (AP) and horseradish peroxidase (HRP) are the most commonly used enzymes and are linked to IgG or antigen by glutaraldehyde or NaIO_4 . The method can be used as a rapid screening device visually or quantitated by a spectrophotometer. The methodology can be automated and adapted to centrifugal analyses and can be performed without the need for a separation step in some cases (Homogeneous assay technique). Kinetic analysis of enzymatic activity may allow increased accuracy and broader applications.

Two recently written reviews^(53,54) have covered discussion of principles, methods, and applications very extensively.

REFERENCES

1. Vincent, M.H. and Bellot, M. Observations a l'occasion du proces-verbal. Diagnostic de la méningite cérébro-spinale à méningocoques par la "précipito-réaction". Bull. Acad. Med. (Paris) 61:326-332, 1909.
2. Dochez, A.R. and Avery, O.T. The elaboration of specific soluble substance by pneumococcus during growth. J. Exp. Med. 26:477-493, 1917.
3. Williams, C.A., Jr. and Grabar, P. Immuno-electrophoretic studies on serum proteins. I. The antigens of human serum. J. Immunol. 74:158-169, 1955.
4. Bussard, A. Description d'une technique combinant simultanément l'électrophorèse et la précipitation immunologique dans un gel: l'électrosynérèse. Biochim. Biophys. Acta 34:258-260, 1959.
5. Gocke, D.J. and Howe, C. Rapid detection of Australia antigen by counterimmuno-electrophoresis. J. Immunol. 104:1031-1032, 1970.
6. Edwards, E.A. Immunological investigations of meningococcal disease. I. Group-specific Neisseria meningitidis antigens in the serum of patients with fulminant meningococcemia. J. Immunol. 106:314-317, 1971.
7. Dorff, G.J., Coonrod, J.D. and Rytel, M.W. Detection by immuno-electrophoresis of antigen in sera of patients with pneumococcal bacteremia. Lancet 1:578-579, 1971.
8. Gordon, M.A., Almy, R.E., Greene, C.H. and Fenton, J.W., Jr. Diagnostic mycoserology by immuno-electro-osmophoresis: A general, rapid, and sensitive microtechnique. Am. J. Clin. Path. 56:471-474, 1971.

18. Rytel, M.W., Dee, T.H., Ferstenfeld, J.E. and Hensley, G.T.
Possible pathogenetic role of capsular antigens in fulminant pneumococcal disease with disseminated intravascular coagulation (DIC). Am. J. Med. 57:889-896, 1974.
19. Dee, T.H., Schiffman, G., Sottile, M.I. and Rytel, M.W.
Immunologic studies in pneumococcal disease. J. Lab. Clin. Med. 89:1198-1207, 1977.
20. Shackelford, P.G., Campbell, J. and Feigin, R.D. Countercurrent immunoelectrophoresis in the evaluation of childhood infections. J. Peds. 85:478-481, 1974.
21. Pollack, M. Significance of circulating capsular antigen in Klebsiella infections. Infect. Immun. 13:1543-1548, 1976.
22. McCracken, G.H., Jr., Sarff, L.D., Glode, M.P., Mize, S.G., Schiffer, M.S., Robbins, J.B., Gotschlich, E.C., Ørskov, I. and Ørskov, F. Relation between Escherichia coli K1 capsular polysaccharide antigen and clinical outcome of neonatal meningitis. Lancet ii:246, 1974.
23. Hibrawi, H., Garrison, F.D. and Smith, H.J. A comparison of various agarose preparations in a counter-immunoelectrophoresis (CIE) system for assaying urinary myoglobin. J. Immunol. Methods 14:59-63, 1977.
24. Tripodi, D., Kochesky, R., Lyons, S. and Davis, O. Immunochemistry of counterimmunoelectrophoresis and the effect of electroendosmotic flow on reactivity. J. Immunol. Methods 4:1-10, 1974.
25. Lund, E. Laboratory diagnosis of pneumococcus infections. Bull. WHO 23:5-13, 1960.

26. Engvall, E. and Perlmann, P. Enzyme linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochem.* 8:871-874, 1971.
27. van Weeman, B.K. and Schuurs, A.H.W.M. Immunoassay using antigen-enzyme conjugates. *FEBS Letters* 15:232-236, 1971.
28. Nakane, P.K. and Pierce, G.B. Enzyme labeled antibodies: Preparation and application for the localization of antigens. *J. Histochem. Cytochem.* 14:929-931, 1967.
29. Avrameas, S. and Uriel, J. Méthode de Marquage d'antigènes et d'anticorps avec des enzymes et son application en immunodiffusion. *Compt. Rend.* 262:2543, 1966.
30. Tew, J.G., Burmeister, J., Greene, E.J., Pflaumer, S.K. and Goldstein, J. A radioimmunoassay for human antibody specific for microbial antigens. *J. Imm. Methods* 14:231-241, 1977.
31. Strange, R.E. and Hambleton, P. An indirect immunoradiometric assay of microbes. *J. Gen. Microbiol.* 93:401-404, 1976.
32. Robern, H., Dighton, M., Yano, Y. and Dickie, N. Double-antibody radioimmunoassay for staphylococcal enterotoxin C₂. *Appl. Microbiol.* 30:525-529, 1975.
33. Boroff, D.A. and Shu-Chen, G. Radioimmunoassay for type A toxin of Clostridium botulinum. *Appl. Microbiol.* 25:545-549, 1973.
34. Catt, K. and Tregear, G.W. Solid-phase radioimmunoassay in antibody-coated tubes. *Science* 158:1570-1573, 1967.
35. Boenisch, T. Improved enzymeimmunoassay for trace proteins in protides of the biological fluids 24th colloquium, Peeters, H., ed., p. 743-749, Pergamon Press, New York 1976.

36. Engvall, E., Jonsson, K. and Perlmann, P. Enzyme linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of an enzyme labeled antigen and antibody coated tubes. *Biochim. Biophys. Acta* 251:427-434, 1971.
37. Nakane, P. K. and Kawaoi, A. Peroxidase labeled antibody. A new method of conjugation. *J. Histochem. Cytochem.* 22:1084-1091, 1974.
38. Cawley, L. P., Minard, B. J. and Gillissen, J. A. Enzyme-linked immunosorb assay (ELISA). Paper presented at the American Society of Clinical Pathologists Workshop on ELISA, 7-9 September 1977, Chicago, Illinois 60612.
39. Ruitenberg, E.J., Steerenberg, P.A., Brosi, B.J.M. and Buys, J. Serodiagnosis of Trichinella spiralis infections in pigs by enzyme-linked immunosorbent assays. *Bull. WHO* 51:108-109, 1974.
40. Avrameas, S. and Ternynck, T. Peroxidase labeled antibody and Fab conjugates with enhanced intracellular penetration. *Immunochem.* 8:1175-1179, 1971.
41. Kato, K., Hamaguchi, Y., Fukui, H. and Ishikawa, E. Enzyme-linked immunoassay. Conjugation of rabbit anti(human immunoglobulin G) antibody with β -D-galactosidase from Escherichia coli and its use for human immunoglobulin G assay. *Eur. J. Biochem.* 62:285, 1976.
42. Saunders, G.C. and Clinard, E.H. Rapid micromethod of screening for antibodies to disease agents using the indirect enzyme-labeled antibody test. *J. Clin. Microbiol.* 3:604-608, 1976.
43. Saunders, G.C. and Wilder, M.E. Disease screening with enzyme-labeled antibodies. *J. Infect. Dis.* 129:362-364, 1974.

44. Saunders, G.C. and Bartlett, M.L. Double antibody solid phase enzyme immunoassay for the detection of staphylococcal enterotoxin A. Report LA-UR 77-285 Revised, Los Alamos Scientific Laboratory, Los Alamos, N.M. 87545. Submitted to: Applied and Environmental Microbiology.
45. Ruitenbergh, E.J., Steerenberg, P.A., Brosi, B.J.M. and Buys, J. Reliability of the enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of Trichinella spiralis infections in conventionally raised pigs. J. Immunol. Methods 10:67-83, 1976.
46. Voller, A., Bidwell, D., Hultdt, G. and Engvall, E. A microplate method of enzyme-linked immunosorbent assay and its application to malaria. Bull. WHO 51:209-211, 1974.
47. Voller, A., Bidwell, D.E. and Bartlett, A. A serological study of human Trypanosoma rhodesiense infections using a microscale enzyme-linked immunosorbent assay. Tropenmed. Parasitol. 26:247-251, 1975.
48. Ruitenbergh, E.J. and Buys, J. Application of the enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of human African trypanosomiasis (sleeping sickness). Am. J. Trop. Med. Hyg. 26:31-36, 1977.
49. Veldkamp, J. and Visser, A.M. Application of the enzyme-linked immunosorbent assay (ELISA) in the serodiagnosis of syphilis. Brit. J. Vener. Dis. 51:227-231, 1975.
50. Hultdt, G., Lagerquist, B., Phillips, T., Draper, C.C. and Voller, A. Detection of antibodies in schistosomiasis by enzyme-linked immunosorbent assay (ELISA). Annals of Trop. Med. Parasitol. 69:483-488, 1975.

51. Russell, H., Facklam, R.R. and Edwards, L.R. Enzyme-linked immunosorbent assay for streptococcal M protein antibodies. J. Clin. Microbiol. 3:501-505, 1976.
52. Wolters, G., Kuijpers, L., Kacaki, J. and Schuurs, A. Solid-phase enzyme-immunoassay for detection of hepatitis B surface antigen. J. clin. Path. 29:873-879, 1976.
53. Scharpe, S.L., Cooreman, W.M., Blomme, W.J. and Laekeman, G.M. Quantitative enzyme immunoassay: Current status. Clin. Chem. 22:733-738, 1976.
54. Wisdom, G.B. Enzyme-immunoassay. Clin. Chem. 22:1243-1255, 1976.

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